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The question waiting to be asked: Innate immunity receptors in the perspective of zoological research

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A b s t r a c t . In the last decade a great effort has been devoted in animal evolutionary ecology to searching for interindividual and interspecific differences in anti-parasite resistance. Although many examples of variability in health-related traits were described in natural animal populations, our knowledge about the underlying genetic features determining this variance is only limited. It has been shown in numerous examples in laboratory animals, domestic animals and humans that variation in the Major histocompatibility complex (MHC) is unable to explain all known genetically determined immunological variation in animals. Still MHC is so far the only gene cluster studied in ecological immunology of free-living animals. In this review we therefore map the potential importance of another group of immunity genes, the Toll-like receptors (TLRs). These innate immunity receptors belong among the most essential components of animal pathogen-recognition system and being reasonably polymorphic they might be responsible for substantial part of variation in disease-resistance in animals.

Key words: animal immunogenetics, ecological and evolutionary immunology, immunity genes, parasites, wild-living populations, ecoimmunology, immunoecology

Introduction

Description of conspecific differences in immune function efficiency has been pivotal for immunological research almost since the establishment of immunology as a science. In zoological sciences, by contrast, there was much interest for a long time neither in animal immune system as a whole, nor in the conspecific or interspecific variability in disease susceptibility in particular. Although a lot of effort has been devoted to research of parasite evolution much less attention has been paid to the response of their hosts. It was not until the influential articles by H a l d a n e (1949) and more recently H a m i l t o n & Z u k (1982) that brought the concept of health and disease into evolutionary biology and animal ecology. Since then parasites are viewed as a principal component of organic evolution. The selective forces posed by parasites modulate various traits of their hosts, including host population genetic structure, population dynamics, life histories, mating systems, sexual dimorphism etc. (C l a y t o n & M o o r e 1997). Although it is apparent that the negative effects of parasites are eliminated by functional immune system it took more than another decade till both theoretical models and experimental research in animal evolutionary ecology focused on the immune activity. Then many zoologists became fascinated by the very similar questions as their colleagues working in immunology. Why some individuals recover after the emergence of a disease and are thus capable to add their genes to the pool of next generations while others die without leaving any offspring? What is the nature of selection on health-

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determining traits? Could we possibly predict the population dynamics or changes in allele frequencies in time and space? The newly arisen ecological and evolutionary immunology has become one of the most rapidly developing zoological disciplines. Nevertheless, although recent research has improved our understanding much (see e.g. Ardia & Schat 2008), our knowledge remains only limited, especially when the functional and genetical basis of animal health variability are concerned.

It was probably the idea of paramount importance of acquired immunity in disease resistance together with the great polymorphism in these molecules that attracted most of the attention of ecological researchers to Major histocompatibility complex (MHC). Because both MHC class I (Salter 2005) and class II (Chow et al. 2005) molecules are important components of the adaptive arm of the immune system, numerous studies aimed to assess the conspecific variability in these glycoproteins and show its ecological and evolutionary consequences (see e.g. Hawley et al. 2005, Westerdahl et al. 2005; also reviewed in Ardia & Schat 2008). Considering the complexity of the vertebrate immune system, it is, however, interesting that there are no ecoimmunological studies focusing on non-MHC genes.

This lack of knowledge was recently pointed out by Acevedo-Whitehouse & Cunningham (2006) who posed in their article an easy question: "Is MHC enough for understanding wildlife immunogenetics?" Apparently, the answer is no. In their study of adult monozygous and dizygous twins in Western Africa Jepson et al. (1997) have shown that more than half of the genetic variability responsible for differences in responsiveness to *Plasmodium falciparum* and *Mycobacterium tuberculosis* antigens may be attributed to non-MHC genes. It is likely that in other pathogens the situation will be very similar.

Thus there are still many questions waiting to be asked. One of them concerns the innate immunity receptors and their role in animal evolutionary ecology. Since the discovery of *Drosophila* Toll gene homologues in vertebrates (Medzhitov et al. 1997) a great attention has been paid in immunological, medical and veterinary research to the investigation of various innate immunity components. In numerous examples it has been shown that it is the innate immunity that is fundamental for successful survival and this is particularly true for innate immunity receptors because these are essential for recognition of any potential danger. To encourage the development of zoological research devoted to the investigation of innate immunity in wild-living animals we present here a brief review of variability found in innate immunity pathogen-reception system. We focus mainly on the most extensively studied group of innate immunity receptors, the Toll-like receptors (TLRs). However, it should be mentioned, that TLRs form only one family of innate immunity receptors and that the others, currently less known, may be of a comparable importance. We also focus mostly on vertebrates because there has been, especially thanks to human medical science, thus far much more information collected about these receptors and consequences of their genetic polymorphism.

Why is adaptive immunity not enough?

Adaptive immunity developed in jawed vertebrates about 500 million years ago as one of their unique features (Danilova 2006, Pancer & Cooper 2006). This part of immunological defence is based on lymphocytes, T cells and B cells, that are able to recognize the presence of non-self structures by their T cell or B cell receptors (TCRs and BCRs) respectively. These receptors are extremely variable. Being composed during the

development of the cells by complex gene rearrangement and gene conversion over 10^{11} types of the receptors are generated. Each lymphocyte clone thus expresses a unique receptor capable to recognize different antigenic structures (presented on MHC molecules in the case of TCRs in T cells). Although there is a strict selection against autoreactive cells, still there is a great risk of self destruction by some particular lymphocyte clones. Any uncontrolled autoimmune reaction would lead to harmful immunopathology that could have fatal consequences for the organism. Therefore, the adaptive immunity is precisely regulated. To be activated, lymphocytes need to obtain additional stimulatory signals through other receptors than their TCR or BCR, originated mostly from cells with no TCR and BCR expression (Viertlboeck & Gobel 2008). Moreover, due to selective proliferation of appropriate lymphocyte clones and adaptive maturation of the B cells it takes up to one week till the adaptive effector mechanisms are ready to fight a new infection. The question then arises which are the components of immunity that co-activate the T and B cells and what protects the host organism before the adaptive immunity is ready to act.

Although effective in defeating pathogens, adaptive mechanisms have never replaced completely the former, so called innate, immunological mechanisms (Danilova 2006). Rather another storey was added to the entirety of the immunity building. Thus, in vertebrate bodies there are much older receptors than TCR and BCR that are common in their structure to both deuterostomes and protostomes (Takeda et al. 2003). Contrary to the adaptive immunity receptors, the innate immunity receptors are capable to recognize pathogens on the basis of conservative structures related to disease and thus signalise the parasite presence directly after the infection appears. This activates immunological mechanisms that are prepared to protect the body immediately and it also enables transduction of additional signals to adaptive immune cells.

The origin and diversity of TLRs

TLRs belong to the basic and presumably also evolutionary most original components of animal pathogen-recognition system (Rich 2005). It was proposed that the TLR family has diverged as early as during, or even before, the Cambrian period (Roach et al. 2005). Receptors belonging among TLRs were described in a variety of animals from vertebrates (see e.g. Roach et al. 2005) to nematodes (Pujol et al. 2001), crustaceans (Inamori et al. 2000), insects (Hoffmann 2003), urochordates (Azumi et al. 2003) and amphioxus (Huang et al. 2008). Recent findings in Cnidaria supported the hypothesis that TLRs are even common to all eumetazoans, i.e. they are more than 600 million years old (reviewed in Leulier & Lemaitre 2008). Moreover, proteins structurally related to TLRs can be found even in amoebae (Chen et al. 2007) and plants (Dangl & Jones 2001) and in many cases they are involved in immunity-associated functions, which indicates even older origin of the structural components of these genes and their conservative function (see Palsson-McDermott & O'Neill 2007).

In total there are currently 23–25 distinct TLR family members known in vertebrates (e.g. Hughes & Piontkivska 2008; it should be, however, noted that the appellation of individual TLRs in different species is rather inconsistent as there is no nomenclature committee for these molecules, see Temperley et al. 2008). According to their phylogenetic proximity TLRs can be divided into six to nine subfamilies (Roach et al. 2005; see Table 1) clustered into two ancient groups that arose by gene duplication prior to the divergence of protostomes and deuterostomes (Hughes & Piontkivska 2008).

Not all TLRs are present in all species. Each vertebrate species studied to date is typically equipped with no more than about a dozen of them (Roach et al. 2005). For instance, in humans there are 10 functional TLRs, in mouse 12, in chicken 10 and in fugu fish 11 known up to now (Hughes & Piontkivska 2008, Roach et al. 2005). Some of them are common virtually to all vertebrates (such as TLR1/6/10, 2, 3, 4, 5, 7) while others are restricted only to some single vertebrate lineages. TLR11 is functional only in mouse whereas human TLR11 is non-functional due to the insertion of a stop codon (Zhang et al. 2004). Moreover, mouse possesses TLR12 while its *Tlr10* gene is disrupted and thus non-functional. TLR15 seems to be unique to chicken and TLR21 is present only in non-mammalian species, i.e. birds and fish. These few most interesting examples illustrate the variability among species in their TLR repertoire and also indicate the diverse function of these receptors. Each vertebrate evolutionary branch has its unique set of TLRs presumably optimised to the defence against its own parasites (see Table 1).

TLRs immunological function

TLRs belong to a group of receptors called Pattern-recognition receptors (PRRs). In vertebrates, contrary to *Drosophila* Toll (Weber et al. 2003), the ligands of PRRs are largely invariant structural components of the parasite cells (called Pathogen-associated molecular patterns; PAMPs) as well as endogenous danger-signalling molecules (termed as Damage-associated molecular patterns; DAMPs; Lotze et al. 2007). PRRs seem to be evolutionary optimised for the detection of most of the pathogen taxa that have co-evolved with vertebrates. As PAMPs are usually basic structural components of the parasite body (nucleic acid, cell wall components in bacteria or envelope protein in viruses; Akira et al. 2006, see also Table 1) their expression cannot be easily avoided. Detection of DAMPs (as e.g. heat-shock proteins), besides, signalizes to the immune system disruption of the organism integrity, which is the first sign of any parasite incursion (Dembic 2005). TLRs are therefore among the very first structures to register parasitic infection in the body.

Individual members of the TLR family differ significantly in their structure. Especially the binding site evolved in each member to recognize different danger-related ligands. Thus, for instance, TLR2 is involved in microbial peptidoglycan detection, TLR4 serves for recognition of lipopolysaccharide (LPS) molecules, TLR5 is responsible especially for flagellin detection and TLR3 and TLR7 bind viral RNA (Akira et al. 2006; for detailed list of TLRs ligands see Table 1). It has been shown in various species of mammals, birds and fish with known TLRs that the ligand molecules are evolutionary broadly conservative for most of the basic TLRs (Iqbal et al. 2005, Tsujita et al. 2004, Schwarz et al. 2007, Keestra & van Putten 2008, Vinkler et al. 2009), although it is perhaps not always the rule (Iliev et al. 2005) and the resultant response may be species-specific (Keestra et al. 2008). The repertoire of TLRs and other PRRs create receptor system that is able to detect most of the infections invading the body. As the signalisation cascades triggered by individual TLRs differ in the involvement of various intracellular signalling molecules, there is opportunity for complex integration of the signals from different PRRs which leads to functional variability in the response of the cell. The response consists of pathogen-specific cell activation resulting in changes in surface molecule and cytokine expression pattern as well as in maturation of the effector mechanisms. Being activated, innate immunity cells are potent to protect the body effectively and are also able to provide co-activating signals to cells of the adaptive arm of immunity, i.e. lymphocytes.

The regulation of the cytokine environment in which lymphocytes are stimulated by their antigens is crucial as it determines the type of the adaptive immune response (either cellular or humoral). This point is critical for the anti-parasite resistance since the activation of wrong

Table 1. Vertebrate TLRs. To each TLR the type of parasites detected and some basic ligands are listed. Included is also information about vertebrate taxa in which each TLR is found. Based on surveys by Roach et al. (2005) and Temperley et al. (2008) with additional evidence about TLRs in individual taxa from Baoprasertkul et al. (2007), phylogeny from Hughes & Piontkivska (2008) and information on ligands in mammals from Akira et al. (2006). Lamprey TLRs (Ishii et al. 2007) are not included.

Receptor	Subfamily	Parasite type	Lignad	Species
TLR1	1	Bacteria	Triacyl lipopeptides	All vertebrates, chicken 2, *Xenopus* 3
TLR2	1	Bacteria	Peptidoglycan, porins, lipoarabinomannan	All vertebrates
		Fungi	Phospholipomannan	
		Protozoa	tGPI-mutin see also TLR1 and 6	
		Viruses	Hemagglutinin protein see also TLR1 and 6	
TLR3	3	Viruses	dsRNA	All vertebrates
TLR4	4	Bacteria	LPS	All vertebrates, but lost in most fish
		Fungi	Mannan	
		Protozoa	Glycoinositolphospholipids	
		Viruses	Envelope proteins	
		Host	Hsp 60 and 70, fibrinogen	
TLR5	5	Bacteria	Flagellin	All vertebrates
TLR5S	5	Acute phase protein		*Xenopus*, fish
TLR6	1	Bacteria	Diacyl lipopeptides, lipoteichoic acid,	Some mammals
		Fungi	zymosan	
TLR7	7	Viruses	ssRNA	All vertebrates
TLR8	7	Viruses	ssRNA	Most vertebrates, pseudogene in chicken
TLR9	7	Bacteria	CpG-DNA	Most vertebrates, not in chicken
		Viruses	DNA	
TLR10	1	Unknown	Unknown	Mammals
TLR11	11	Bacteria	Unknown	Mouse
		Protozoa	Profilin-like molecule	
TLR12	11	Bacteria	Profilin	Mouse
TLR13	11	Unknown	Unknown	Some mammals, *Xenopus*
TLR14	1	Unknown	Unknown	*Xenopus*, fish
TLR15	15	Bacteria	Unknown	Chicken
TLR16	16	Unknown	Unknown	Xenopus
TLR18	18	Bacteria	Unknown	Fish
TLR19	11	Unknown	Unknown	Fish
TLR20	11	Bacteria	Unknown	Fish
TLR21	11	Unknown	Unknown	Chicken, *Xenopus*, fish
TLR22	11	Bacteria	Unknown	*Xenopus*, fish
TLR23	11	Unknown	Unknown	Fish

effector mechanisms does not lead to infection clearance and may have even an adverse effect on health (e.g. in the form of allergic reaction). Moreover, TLRs may act as antigen binding molecules directly responsible for antigen internalisation prior its presentation on the MHC surface (Dembic 2005). TLRs therefore influence even the spectrum of antigens against which lymphocyte response may be targeted. Altogether, due to the variance in TLRs the innate immune system is able to initiate the signalization directing the quality as well as quantity of the innate and adaptive immune response (Dembic 2005). Thus, TLRs represent a real bridge between mechanisms of innate and adaptive immunity enabling their combined action (see e.g. Akira et al. 2001).

Molecular structure of TLRs

As TLRs serve as ligand binding molecules, the tertiary structure is highly important for their correct function. TLRs form a family of type I transmembrane glycoproteins that typically consists of a ligand binding domain composed of several leucine-rich repeat (LRR) motives and a cytosolic signal-mediating Toll-Interleukin-1 receptor (TIR) domain. Although many TLRs are transmembrane proteins with their binding sites opened to the cell surface (TLR1, 2, 4, 5 and 6), others (TLR3, 7, 8 and 9) are expressed almost exclusively into the intracellular compartments such as endosomes (Akira et al. 2006). In fish and amphibians a modified short TLR5 (TLRS5) was found which even lacks its TIR domain and being soluble serves as an acute-phase protein (Roach et al. 2005, Tsukada et al. 2005). It has been shown that while most of the molecular surface of the TLR exodomain is masked by glycosylation, the ligand-binding region remains glycosylation-free (Bella et al. 2008).

In many TLRs we have no idea about the form in which they detect their ligands. In those in which we know at least something it seems that in many cases the TLR is associated with other molecules forming an activation cluster (Triantafilou & Triantafilou 2005). For instance TLR4 associates in LPS binding with CD14 and some other accessory molecules (Kim et al. 2007). It seems that some TLRs, such as TLR3 (Bell et al. 2005, Choe et al. 2005) are able to form homodimers. This might have been a step towards diversification of these dimeric structures to improve the pathogen detection. Mammalian TLR1, TLR6 and TLR10 were shown to be able to form heterodimers with TLR2 creating functional receptors for a great variety of PAMPs (Jin et al. 2007). Similar feature has been recently described also for related chicken TLR2-1 and TLR2-2 (Higuchi et al. 2008; for corrected names see Temperley et al. 2008). Thus, combination of the TLRs in the dimeric structure enlarges the spectrum of pathogen structures detected. To predict correctly the ability of an individual to detect the potential infection and subsequently respond to it, it is therefore useful to map all molecules participating in the parasite recognition.

Searching for the origin of health

As TLRs belong among the first elements able to detect potential danger and initiate the immune response, their importance for successful parasite defeat is crucial. It is therefore not surprising that TLRs are exposed to strong selection. Interspecific comparisons of *Tlr* genes revealed that especially cytoplasmic TIR domain is highly conservative due to purifying selection (Smirnova et al. 2000, Vinkler et al. 2009). The TIR domain which is responsible for signal transduction within the cell is required to remain unchanged in its structure as otherwise its interaction with other molecules in the signalling cascade would be

impaired. This is, however, not the case of the extracellular ligand-binding domain which is fare more variable, both on interspecific and conspecific level.

Although it was rather unexpected, considering the conservatism of the detected structures, TLRs were found polymorphic even within species and populations. It has been shown in laboratory mice that in 35 common strains there are 22 *Tlr4* alleles, 13 of which create amino-acid substitutions and thus potentially alter also the binding features of the receptor (S m i r n o v a et al. 2000). Similarly, in 6 domestic chicken inbred lines 14 coding nucleotide substitutions were identified, five of which resulted in amino-acid changes harboured in LRR binding regions (L e v e q u e et al. 2003). Remarkable synonymous as well as non-synonymous polymorphism in various TLRs genes has been recently described also in some domesticated species, e.g. in sheep (Z h o u & H i c k f o r d 2008). The TLRs polymorphism is, however, not restricted only to inbred lineages of laboratory animals and domestic animals that are exposed to artificial selection. The results of S t e p h a n et al. (2007) indicate that wild-living animals might exhibit even greater variety of natural genetic polymorphism in these genes. In six wild-derived inbred strains of mice they found substantial polymorphism in *Tlr3*, *Tlr4* and *Tlr9*. In TLR3 also the functional significance of the genetic change was demonstrated. Finally, extensive TLRs polymorphism has been found also in humans, the only free-living animal species studied up to now. The human TLRs polymorphism was documented to be reasonably common. In a Chinese population, for instance, non-synonymous polymorphism was found in *Tlr3*, *Tlr7*, *Tlr8* and *Tlr9* genes with minor allele frequencies ranging from 3% up to 44% (C h e n g et al. 2007). It is also remarkable that different populations vary significantly in the frequencies of individual alleles (T e x e r e a u et al. 2005, C h e n g et al. 2007, F e r w e r d a et al. 2007).

It has been shown on numerous examples that allelic variants of TLRs may be associated with changes in resistance to various parasite infections and diseases occurrence. In humans, there is, for example, association of polymorphism in TLR2 with resistance to urinary tract infections, leprosy and tuberculosis (T e x e r e a u et al. 2005, T a b e l et al. 2007, B o c h u d et al. 2008). TLR4 alleles alter the probabilities of Crohn's disease, gastritis and ankylosing spondylitis appearance (A c h y u t et al. 2007, H u m e et al. 2008, P o i n t o n et al. 2008). Viral receptor TLR3 associates with nasopharyngeal carcinoma risk (H e et al. 2007), TLR7 with differences in the immune response to hepatitis C virus (S c h o t t et al. 2008) and TLR8 with differential resistance to viral diseases, such as HIV (O h et al. 2008). Risky might be also certain combinations of alleles in several receptors; e.g. TLR4 and TLR9 polymorphism alters susceptibility to pulmonary aspergillosis (C a r v a l h o et al. 2008). The existence of the polymorphism is often being viewed as the emergence of negative mutations from a standard genotype. This interpretation seems, however, incorrect as in many cases the minor alleles of TLRs are highly beneficial. The impaired function of the receptors may be in some cases adaptive as it lowers the risk of autoimmune diseases (H e r n e s n i e m i et al. 2008). Several TLR4 alleles are known, for instance, to reduce risk of inflammatory illnesses such as atherosclerosis (G i b s o n et al. 2008), periodontitis (J a m e s et al. 2007) or myocardial infarctions (E n q u o b a h r i e et al. 2008) in humans. Similarly, some TLR alleles may also decrease the probability of the development of allergic illnesses (P r e s c o t t et al. 2008); e.g. TLR2 polymorphism has been found to correspond to atopic dermatitis (N i e b u h r et al. 2008), certain TLR4 alleles are associated with lower hay fever atopy occurrence (S e n t h i l s e l v a n et al. 2008) and minor alleles of TLR1, 6, 9 and 10 have been shown to mediate protective effects on atopic asthma (K o r m a n n et al. 2008, L a c h h e b et al. 2008). Although the regulation of the immune response may

be changed importantly (see e.g. Hodgkinson et al. 2008, Mrabet-Dahbi et al. 2008, Roelofs et al. 2008, Wochrle et al. 2008), usually there are alternative pathways to balance the immune protection (Gu et al. 2008), which lowers the negative effect of the impaired TLR signalization on health.

We have mentioned minor alleles causing decreased immunological responsiveness. Nevertheless, in many cases minor alleles are associated on the contrary with stronger responsiveness and enhanced protection to parasite infections. Dhiman et al. (2008), for example, have described several alleles in various TLRs that are associated with increased responsiveness to measles vaccination. Moreover, it has been shown by Hawn et al. (2005) that some allelic variants of TLR4 are associated with increased resistance to Legionnaires disease and Ferwerda et al. (2007) have recently described a protective effect of TLR4 polymorphism against malaria. Also in domestic chicken evidence suggests that minor allele variability in TLR4 is linked to differences in resistance to *Salmonella* infection (Leveque et al. 2003). Although PAMPs recognized by TLRs are rather conserved, still they significantly differ in structure across the pathogen spectra and even among different strains of the same parasite species (Zdorovenko et al. 2007, Dentovskaya et al. 2008). Given the host-parasite co-evolution, this variability may be associated with structural changes in TLRs binding regions in evolutionary distinct lineages of hosts (Kubarenko et al. 2007). Although individual alleles decrease the risk of some diseases they may on the other hand increase the susceptibility to others. It therefore seems that TLRs are exposed to either positive or balancing selective pressure, depending on particular geographic population (Chen et al. 2008, Ferrer-Admetlla et al. 2008) and that the allelic frequencies are dependent on the epidemiological context of the population (Ferwerda et al. 2007). These examples clearly show that at least some polymorphism in innate immunity receptors, such as TLRs, may have either beneficial or deleterious effect on the disease outcome, depending on the pathogen concerned.

Conclusion

During the decade since the TLRs were described we have learned relatively much about them. We are aware that they are not the only innate immunity receptors important to animal health protection (as there are also many other receptors, such as NOD-like receptors, RIG-I-like receptors or C-type lectin receptors; Palsson-McDermott & O'Neill 2007) and we already understand fairly well their immunological role. We have also gained some basic information about their variability in laboratory and domestic animals as well as in human populations and in recent years immunological research is showing us how this polymorphism is related to disease resistance. However, what we still miss is virtually any idea about the polymorphism in innate immunity genes in wildly living animal populations. These populations are typically exposed to selective forces mediated by very different parasites and in very different ecological contexts. Has this environmental variety led to specific adaptations in the innate immunity system across the animal kingdom? It is more than probable, based on the information reviewed herein, that the answer is yes. Moreover, not only the polymorphism in structure but also the one in expression may influence the resultant adaptiveness of the immune response after the pathogen incursion (Bilodeau-Bourgeois et al. 2008). Eventually, also the signalling cascades regulation might be modified to fit the most commonly met diseases. Even though less information about the expression and regulation of

TLR signalling is so far available, these aspects should be focused in future to give us more comprehensive answers. We believe that the immunogenetics of natural animal populations may offer us important new insights into the general aspects of host-parasite co-evolution. Positively, TLRs are good models to start with.

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Appendix. Abbreviations used.

BCR	B cell receptor
DAMP	Damage-associated molecular pattern
LRR	Leucine-rich repeat
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
NOD	Nucleotide binding and oligomerization domain
PAMP	Pathogen-associated molecular pattern
PRR	Pattern-recognition receptor
RIG-I	Retinoic acid-inducible gene 1
TIR domain	Toll-Interleukin-1 receptor domain
TLR	Toll-like receptor
TCR	T cell receptor

Genes are written in small letters and italics (e.g. *Tlr4*).